REMARKS

Reconsideration of the Examiner's rejection of the present application is requested respectfully in view of the above amendments and the following remarks.

INTERVIEW SUMMARY

Applicants respectfully acknowledge an interview between the Examiner and the below-named attorney on November 2, 2009. The references cited in the present office action were discussed, and applicants' attorney presented reasons why the claims of the present application are patentable over those references. In particular, the unexpected effect on Factor Xa inhibition with the compounds of the present invention were emphasized and distinguished over the cited references, as is discussed further below. Agreement was reached that the claims of the present application are patentable over the cited references.

STATUS OF THE CLAIMS

Claims 1-19 and 25-29 were pending at the time of the present Office Action. Claims 25, 26 and 29 stand withdrawn from consideration. No claims have been amended, added or deleted. Therefore, claims 1-19 and 27-28 are now presented for review. If these claims are found allowable, then rejoinder of the withdrawn claims is respectfully requested.

SUMMARY OF OFFICE ACTION

The specification has been objected to for lacking continuity data. An Application Data Sheet (ADS) is filed herewith, which duly references all of the priority patent applications.

Claims 1-19, 27 and 28 stand rejected under 35 U.S.C. §103(a) as being unpatentable over the combined teachings of Petitou et al. (US 5,378,829) in view of Oosta et al. (of record).

CLAIM REJECTION UNDER 35 U.S.C. §103(a)

Claims 1-19, 27 and 28 stand rejected under 35 U.S.C. §103(a) as being unpatentable over the combined teachings of Petitou et al. (US 5,378,829)in view of Oosta et al. (of record). Applicants respectfully traverse this rejection.

As discussed in previous responses, the claims of the present application are directed to synthetic polysaccharides containing 8 to 24 monosaccharide units, further characterized as set forth therein. As explained in the specification, at page 2, lines 4-9, prior to the present invention short chain oligosaccharides were known, such as those described in US 5,378,829, which is the Petitou reference cited as the primary reference in the present rejection. citation. But as discussed previously, and as noted by the Examiner, Petitou only describes oligosaccharides having a maximum of 7 monosaccharide units, and does not disclose compounds having 8 to 24 saccharide units.

Oosta is cited by the Examiner as a secondary reference to show that various larger size polysaccharides were known in the art, and that larger heparin fragments exhibit greater biological activities that hexasaccharides. The Examiner then indicates that the teachings of Oosta would have motivated one skilled in the art to use the Petitou technique to produce polysaccharides of more than 7 units. Applicants respectfully disagree with this conclusion.

Oosta is a 1980 reference which describes a method of fractionating heparin. As known in the art, the starting material heparin is itself a heterogeneous product, composed of a great number of oligosaccharidic chains differing in their chemical structure as well in their length. According to Oosta, the oligosaccharidic chains naturally occurring in heparin substances are fractionated into mixtures of various length fragments, which are then separated by use of a chromatographic column. At the time of Oosta, such oligosaccharides were made by fractionating natural heparins (particularly pig heparin) rather than by stepwise synthesis. That is, Oosta discusses the biological activities of oligosaccharide fractions isolated from a mixture resulting from chemical depolymerization of heparin (see the first paragraph of the Discussion section on page 832: "We have randomly cleaved preparations of heparin … by using appropriate chemical techniques."), and subsequent separation of fractions with different sizes by gel filtration and affinity fractionation.

Products obtained by such techniques are different from the claimed oligosaccharides, and different from the oligosaccharides synthesized by Petitou. The oligosaccharides of the present invention and those of Petitou are obtained by stepwise synthesis of specific building blocks intermediates. In the present claims and in the claims of Petitou, all of the naturally occurring hydroxyl (-OH) R side groups, attached though the O linkage to the saccharide chain, are either etherified or esterified to respective -O-alkyl or $-O-SO_3^-$ groups. This allows for the synthesis of unique products by the intentional selection of the side groups and their locations on the saccharide chain.

The natural heparin derived fractions obtained by Oosta are heterogeneous products, comprising mixtures of different oligosaccharides having the same length (but varying in their monosaccharidic constituents) as well as the residual presence of shorter or longer chains. The larger the fraction, the higher the heterogeneity of the fraction. For example, in Table 1 of Oosta the fractions of 14 or 16 residues are prefaced by the approximation symbol "~". Even in the lowest fraction (hexasaccharide), hundreds of different compounds may be found, since different sugars may be present and different substitution patterns thereof may occur. Thus, the teachings of Oosta are limited to fractions comprising mixtures of hundreds of components. In contrast, as noted at page 27, lines 13-15 of the present application, all of the compounds described in the present examples are "homogeneous on thin-layer chromatography (TLC) and have spectral properties in agreement with their structure."

As described in Oosta, (which was published over 25 years ago) the functional saccharide fragments discussed therein were all made by random scissioning of long-chain porcine heparin. These random length fragments were then examined for their properties. Not only is there no description, or even a suggestion, that these fragments could be synthesized, but there is not even any teaching of how to accurately extract a particular fragment. There is also no discussion of what monomeric saccharide groups are present, or what side groups are on those saccharides. Since the scissioning was random, the chemistry and properties of the fragments was also random. This may have been useful early research in development of fractionated heparin compositions, but in no way provides any teaching that longer chain fragments, or any fragments for that matter, could be specifically synthesized. As noted above, the saccharides of the present invention are specifically configured to provide a known substrate with known side groups.

In the first full paragraph on page 5 of the present Office Action, the Examiner indicates that Oosta discloses that "…fragments larger than hexasaccharides possess much greater factor Xa and thrombin inhibitory activity than hexasaccharides." This is cited to show that Oosta provides a teaching that longer polysaccharide compounds always provide greater factor Xa activity, and that therefore it would have been obvious for one skilled in the art to lengthen the hexasaccharide chains set forth in Petitou to obtain increased factor Xa inhibition.

However, applicants submit that this generalization is not correct, and does not apply to the compounds of the present invention. Exemplified compounds of the present application were tested for Factor Xa inhibition. The results of these tests were published in an article by Petitou, et al., presented in the "Letters to Nature" section of Nature, volume 398, 1 April 1999, pages

417-422. A copy of this article, which was published after the priority date of this application, is provided herewith as Exhibit A. The authors of the article include Maurice Petitou, Philippe Duchaussoy and Jean-Marc Herbert, who are also named inventors of the present application. Examples 1 to 6 of the present application correspond to Compound Numbers 7 and 2 to 6 of the article. In addition, Compound Number 1 of the Petitou et al article corresponds to a compound of Example 2 of the cited Petitou '829 patent. Details of the synthesis and testing procedures are set forth in the article.

The following table, based on Table 1 of the cited Petitou et al. article presents the Factor Xa inhibition values for Examples 2-6 and 1 of the present application, as well as the Example 2 compound from the cited Petitou '829 patent, for comparison:

Example No.	Compound No. of Article	Number of saccharide units	Factor Xa inhibition (U/mg)
Example 2 of US 5,378,829	Compound No. 1	6	325 ± 16
Example 2 of application	Compound No. 2	10	405 ± 32
Example 3 of application	Compound No. 3	12	360 ± 29
Example 4 of application	Compound No. 4	14	310 ± 16
Example 5 of application	Compound No. 5	16	359 ± 29
Example 6 of application	Compound No. 6	18	270 ± 23
Example 1 of application	Compound No. 7	20	236 ± 19

The data presented in this table show that the Factor Xa inhibition does not merely increase as the fragment length increases. The Factor Xa inhibition is generally higher up to 16 saccharide units, and then starts decreasing for larger fragments. Such results could not have been predicted from the disclosure of the Oosta reference.

For all of these reasons, it is submitted that the invention set forth in the claims of the present application the Examiner is respectfully requested to reconsider and withdraw the present would not have been taught to one skilled in the art by the disclosures of the Petitou et al. '829 patent or the Oosta reference, whether taken individually or combined. Therefore, reconsideration of the rejection of claims 1-19, 27 and 28 under 35 U.S.C. §103(a) over this combination of references is respectfully requested.

It is submitted that the claims in the present application are now in condition for allowance, and action to that effect is respectfully requested.

The Commissioner is hereby authorized to charge any additional fees or credit any overpayment resulting from this Amendment to Deposit Account 18-1982.

Respectfully submitted,

November 4, 2009

/Ronald G. Ort/
Ronald G. Ort, Reg. No. 26,969
Attorney for Applicants

sanofi-aventis U.S. Inc
Patent Department
Route #202-206 / P.O. Box 6800
Bridgewater, NJ 08807-0800
Telephone (908) 231-2551
Telefax (908) 231-2626

EXHIBIT A

Timescales. To measure genus-level completeness we used two alternative stratigraphic subdivisions of the Phanerozoic, developed independently by the two authors. The first was a previously published subdivision[24] using various timescales and conventional stratigraphic series, stages and substages split and lumped to yield 103 units with a mean duration of 5.5 million years (Myr) and approaching minimal variance in duration. The second was a subdivision based mainly on the timescale of ref. 25, variously divided to yield 107 intervals averaging 5.3 Myr in duration, again approaching minimal variance. Although the 107-interval subdivision tends to yield lower estimates of preservation probability (Figs 1–3), the tendency is not statistically significant by a binomial test ($N = 17$ cases in which the two timescales yield different results; two-tailed $P = 0.14$). The dependence of estimated preservation probability on timescale reflects idiosyncrasies in the lumping and splitting of stratigraphic units rather than the average level of resolution, which differs little between the two timescales. With each subdivision, we included all genera in a comprehensive, global database[26,27] whose first and last occurrences could be reliably placed into one of the stratigraphic intervals. Out of 24,190 genera in the taxonomic groups analysed, 16,464 (68.1%) could be resolved with the 103 intervals of geologic time, and 16,338 (67.5%) could be resolved with the 107 intervals.

Data. We used the family data compiled by one of us[26,28], supplemented extensively with data from ref. 29, to measure the proportion of living families with some fossil record. The taxonomy of living families and the number of unfossilized living families are based largely on ref. 30. To limit problems of the 'Pull of the Recent'[9] in the analysis of genera, including the artificial truncation of durations that would affect our estimate of genus preservation probability[6,7], we used only genera that first occur before the Cenozoic era. We excluded groups with less than 100 resolvable fossil genera or less than 10 living families, which, with greater proportional counting error, would yield less stable measures of completeness. Our data thus favour more diverse and better-preserved groups (nemertines, priapulids and many other soft-bodied phyla were excluded, for example), but our measures are not biased for those groups used as we included all resolvable marine taxa within each group. We did not tabulate the proportion of living genera with a fossil record, as adequate data are not available for most groups.

Sample sizes for Fig. 1 (number of living families and number of genera resolved with the 103-interval timescale and the 107-interval scale) are: Anthozoa (122, 1,179 and 1,162); Asterozoa (53, 154 and 156); Bivalvia (103, 833 and 804); Brachiopoda (19, 2,840 and 2,778); Bryozoa (143, 715 and 734); Cephalopoda (41, 3,133 and 3,114); Chondricthyes (42, 173 and 169); Crinoidea (26, 824 and 830); Echinoidea (50, 228 and 241); Gastropoda (230, 885 and 873); Malacostraca (371, 191 and 201); Osteichthyes (328, 361 and 312); Ostracoda (34, 1,072 and 1,073); Polychaeta (75, 123 and 123); and Porifera (107, 590 and 570). Because no asterozoan genera have resolved ranges of three intervals on the 107-interval scale, results are presented for the 103-interval scale only. Porifera excludes Archaeocyatha, which are very short-lived as a group and thus have durations severely truncated by the extinction of the group shortly after its origin. Sample sizes for Figs 2 and 3 (number of genera in 103-interval scale and in 107-interval scale) are: Ammonoidea (2,235 and 2,215); Blastozoa (237 and 237); Coleoidea (127 and 118); Conodonta (261 and 264); Graptolithina (226 and 229); Nautiloidea (781 and 791); Trilobita (2,439 and 2,468); Palaeozoic Chondrichthyes (82 and 83); and Mesozoic Chondrichthyes (91 and 86). To increase sample size for coleoids, genera originating in the Palaeocene and Eocene have been included.

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Correspondence and requests for materials should be addressed to M.F. (e-mail: mfoote@midway.uchicago.edu).

Synthesis of thrombin-inhibiting heparin mimetics without side effects

Maurice Petitou, Jean-Pascal Hérault, André Bernat, Pierre-Alexandre Driguez, Philippe Duchaussoy, Jean-Claude Lormeau & Jean-Marc Herbert*

Sanofi Recherche, 195 route d'Espagne, 31036 Toulouse, France

Unwanted side effects of pharmacologically active compounds can usually be eliminated by structural modifications. But the complex heterogeneous structure of the polysaccharide heparin[1] has limited this approach to fragmentation, leading to slightly better-tolerated heparin preparations of low molecular mass[2]. Despite this improvement, heparin-induced thrombocytopaenia[3] (HIT), related to an interaction with platelet factor 4 (PF4) and, to a lesser extent, haemorrhages[4], remain significant side effects of heparinotherapy. Breakthroughs in oligosaccharide chemistry[5] made possible the total synthesis of the pentasaccharide antithrombin-binding site of heparin[6,7]. This pentasaccharide represents a new family of potential antithrombotic drugs, devoid of thrombin inhibitory properties, and free of undesired interactions with blood and vessel components. To obtain more potent and well-tolerated antithrombotic drugs, we wished to synthesize heparin mimetics able to inhibit thrombin, that is, longer oligosaccharides. Like thrombin inhibition, undesired interactions are directly correlated to the charge and the size of the molecules[8], so we had to design structures that were able to discriminate between thrombin and other proteins, particularly PF4. Here we describe the use of multistep converging synthesis to obtain sulphated oligosaccharides that meet these requirements.

The structures we synthesized were inspired by the structure of heparin itself and designed according to current knowledge of its mechanism of action[9] (Fig. 1). Briefly, heparin binding to antithrombin induces a conformational change in the inhibitory loop of the protein (allosteric activation) resulting in recognition and inhibition of factor Xa. Thrombin is inhibited through a template mechanism (entropic activation): it is electrostatically attracted by heparin and collides with heparin-bound antithrombin. Thus, thrombin inhibition requires a longer heparin chain, more prone to interact with other biological molecules, particularly PF4, than the short pentasaccharide[8]. Natural heparin chains, because of their sulphation profile, cannot discriminate between thrombin and many other proteins (hence the undesired side effects). But chemical synthesis, through the fine tuning of the length and the charge of the synthetic oligosaccharides, should allow us to identify structures able to do so. Our approach was also influenced by our ultimate goal, to obtain a drug substance, which pushed us to keep the chemistry as simple as possible to facilitate scaling up.

The above mechanistic considerations on the anticoagulant action of heparin suggest that the structure of an oligosaccharide molecule able to mimic its full anticoagulant activity would include an antithrombin-binding domain (A-domain) coupled to a thrombin-binding domain[10] (T-domain). Such a heparin sequence comprises between 14 and 20 saccharide units[11–14]. Moreover, from the specific interaction of antithrombin with heparin (Fig. 1c, d), it was inferred that one only of the two possible ways of elongating the A-domain with a T-domain (either at the reducing or at the non-reducing end) should lead to thrombin inhibition[10]. Our first approach towards such molecules, dictated by our desire to use a 'short' synthetic route, circumvented the problem of the relative position of these two domains. We reasoned that an A-domain, because it is negatively charged, can serve as a T-domain, and that a continuum of A-domains would necessarily display the proper relative position of the two domains, because antithrombin could bind at either end of the molecule, and thrombin would be attracted by the remaining part of the chain (Fig. 1). Thrombin inhibition should therefore be observed as soon as the saccharide is long enough to accommodate antithrombin and thrombin at the same time. We worked with the so-called 'non-glycosaminoglycan' series[15], in which *N*-sulphate groups are replaced by *O*-sulphate groups, and hydroxyls are alkylated (Fig. 2). These structural modifications fully preserve the specific binding to antithrombin[16] yet they dramatically simplify the synthesis.

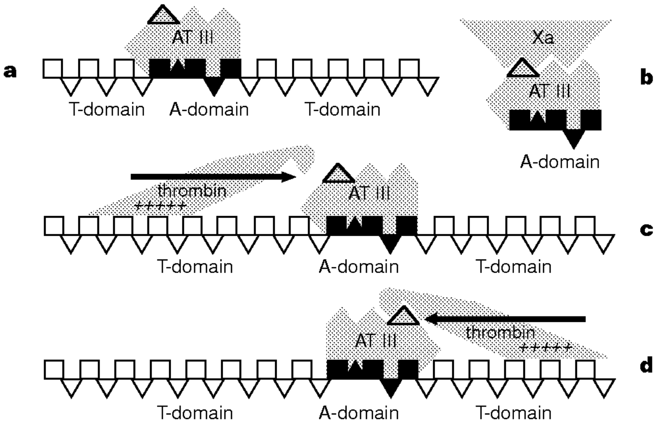

Figure 1 Inhibition of factor Xa and thrombin by antithrombin and heparin. Heparin is shown here (see also Fig. 2) as a repetition of squares (glucosamine units) and triangles (uronic acid units). **a**, Through binding to the antithrombin pentasaccharide-binding-site (A-domain, black squares and triangles), antithrombin conformation changes (hatched triangle) in a manner sufficient to inhibit factor Xa without any contribution from the rest of the saccharidic chain. This is why the pentasaccharide representing the antithrombin-binding site can inhibit factor Xa (**b**). In contrast, thrombin inhibition requires attraction by the negative charges of the thrombin-binding domain (T-domain). Thrombin 'slides' along the heparin chain until it hooks itself on the exposed loop of activated antithrombin. Because the heparin-antithrombin complex has a well-defined structure, thrombin must approach from the correct side (possibly **c** or **d**) to be irreversibly inhibited.

Having identified a highly symmetrical hexasaccharide that can bind to and activate antithrombin, we synthesized larger homologous oligosaccharides and tested their anticoagulant properties[17]. All compounds, because they contain an A-domain, bound to antithrombin and inhibited factor Xa. The 6-, 10-, 12- and 14-mer saccharides were inactive in the thrombin inhibition assay, whereas the 16-, 18- and 20-mer saccharides displayed size-dependent increasing activity in this assay, the 20-mer saccharide being half as potent as standard heparin (Table 1). In an *in vivo* experimental model of venous thrombosis (Wessler model in the rat)[18], in which thrombosis was induced by a combination of a thrombogenic challenge (recombinant tissue factor) and stasis, all compounds displayed a similar, dose-dependent antithrombotic effect related to their specific anti-factor Xa activity (Table 1). In an arterial thrombosis model (arterio-venous shunt in the rat) in which platelets and thrombin have a preponderant role[19], the intravenous administration of the thrombin inhibitory compounds (**5**–**7**) reduced thrombus formation in a dose-dependent manner with half-maximal effective dose (ED$_{50}$) values similar to that of standard heparin, whereas compounds **1**–**6** (devoid of thrombin inhibitory effect) were inactive (Table 1). Unfortunately, all these molecules were neutralized by PF4 and they activated platelets in the presence of plasma from HIT patients (not shown). Thus, despite their antithrombotic properties (the 20-mer saccharide exhibited an antithrombotic activity similar to that obtained for standard heparin), the cross reactivity of these compounds with PF4 prompted us to explore another family of related molecules with a structure closer to that of heparin, that is, possessing a specific A-domain prolonged by a T-domain that is not recognized by antithrombin (Fig. 2).

The above results on thrombin inhibition by compounds **2**–**7** clearly established that the oligosaccharide we are looking for must contain at least 15 or 16 saccharide units (this gives a more precise answer to the long-standing question about the size of heparin fragments able to catalyse thrombin inhibition by antithrombin[11–14]). In contrast to the first approach, an important issue in the design of these structures was how to attach the T-domain at the proper end of the A-domain to obtain efficient thrombin inhibition (Fig. 1). Studies based on modelling of the ternary heparin/antithrombin/thrombin complex[10] and recent crystallography studies[20] suggested that the T-domain may need to be attached at the non-reducing end so, knowing that we had to synthesize at least a 15-mer saccharide, we targeted compounds **8**–**10**. We selected as A-domain a high-affinity analogue of the antithrombin-binding sequence[21] (DEFGH; Fig. 2). Because thrombin binding in the T-domain is mainly a matter of electrostatic attraction of the anion-binding exosite II of the protein by the anionic polysaccharide[22], and in order to keep the chemistry feasible, we used alternating α- and β-linked 3-*O*-methyl-2,6-di-*O*-sulpho-D-glucose units as a mimic of the regular region of heparin. Biological tests performed on these compounds (Table 1) demonstrated that they were inhibitors of both factor Xa and thrombin. Thrombin inhibition was size dependent, which explains the greater ability of a longer negatively charged molecule of heparin to attract thrombin and bring it into contact with antithrombin. The minimum number of saccharide units in the chain that could inhibit thrombin was 15 (compound **8**). Note that the 19-mer saccharide (compound **10**) was as potent as the most active fraction isolated from a standard heparin preparation[23].

We then had to prove that the opposite arrangement of A- and T-domains resulted in an inactive compound. We therefore synthesized the 18-mer saccharide **11** (Fig. 2) made of the same structural elements as **8**–**10** but in which their relative positions have been

Table 1 Biological properties of the synthetic oligosaccharides

Compound number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	Heparin	LMWH
Number of saccharide units	6	10	12	14	16	18	20	15	17	19	18	17	~10–50	~6–30
Molecular mass	2,217	3,606	4,301	4,995	5,690	6,384	7,078	5,618	6,378	7,139	6,698	5,322	~15,000	~4,500
In vitro activities														
Affinity for antithrombin (K_d, nM)	350 ±10	21 ±4.3	19 ±2.9	19 ±2.1	22 ±1.3	28 ±1.4	24 ±1.7	16 ±0.3	3.3 ±0.8	1.2 ±0.2	7.3 ±1.4	7.0 ±1.5	25 ±0.2	n.d.*
Factor Xa inhibition (units mg^{-1})	325 ±16	405 ±32	360 ±29	310 ±16	359 ±29	270 ±23	236 ±19	370 ±9	270 ±8	290 ±29	230 ±16	270 ±8	180	170 ±17
Thrombin inhibition (IC_{50}, ng ml^{-1})	>10,000	>10,000	>10,000	>10,000	130 ±10	23 ±4	6.7 ±3	41 ±3	5.3 ±0.2	1.7 ±0.5	164 ±6.5	5.3 ±0.3	3.3 ±0.5	27 ±
In vivo activities														
Venous thrombosis (ED_{50}, μg kg^{-1}, i.v.)	300 ±70	280 ±49	360 ±62	390 ±45	150 ±21	250 ±20	110 ±17	65.5 ±3	40 ±4	38 ±9	423 ±27	15 ±2	80 ±3	87 ±3
Arterial thrombosis (ED_{50}, μg kg^{-1}, i.v.)	>1,000	>1,000	>1,000	>1,000	520 ±140	610 ±230	620 ±130	380 ±30	570 ±130	770 ±200	>1,000	70 ±16	700 ±90	>1,000

Means ± s.d. ($n = 10$). Affinity for antithrombin, factor Xa inhibition, thrombin, inhibition and anti-thrombotic activities were determined using published procedures[18]. MWH, low-molecular-weight heparin.

* n.d., not determined.

reversed. The biological properties of compound **11** (Table 1) showed that the affinity for antithrombin and the anti-factor Xa activity were in the same range as that observed for compounds **8–10**. However, compound **11** hardly inhibited thrombin in the presence of antithrombin (from 30 to 100 times weaker on a mass basis than the corresponding 17/19-mer saccharides **9** and **10**).

As seen with compounds **5–7**, although compounds **8–10** were potent antithrombin-mediated thrombin inhibitors, their anticoagulant activity was neutralized by PF4, indicating that reducing the size to the minimum that still allowed thrombin inhibition was not sufficient to abolish the undesired interaction with PF4.

We therefore explored how to reduce the charge of the molecule, the second parameter governing nonspecific reactivity of polyanionic compounds[8]. We based the design of our new targets on the

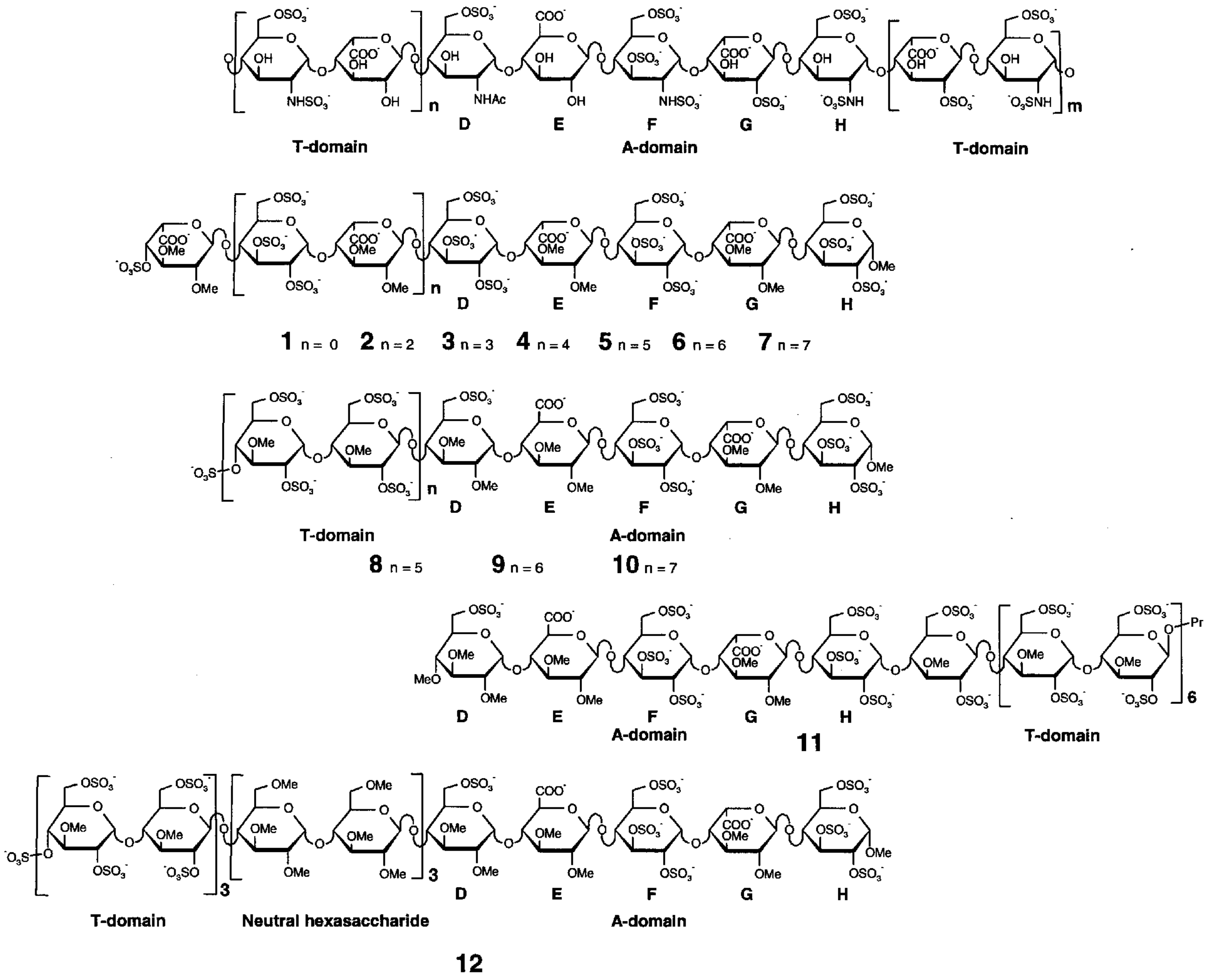

Figure 2 Structure of an anticoagulant heparin molecule (top line) and of oligosaccharides 1–12.

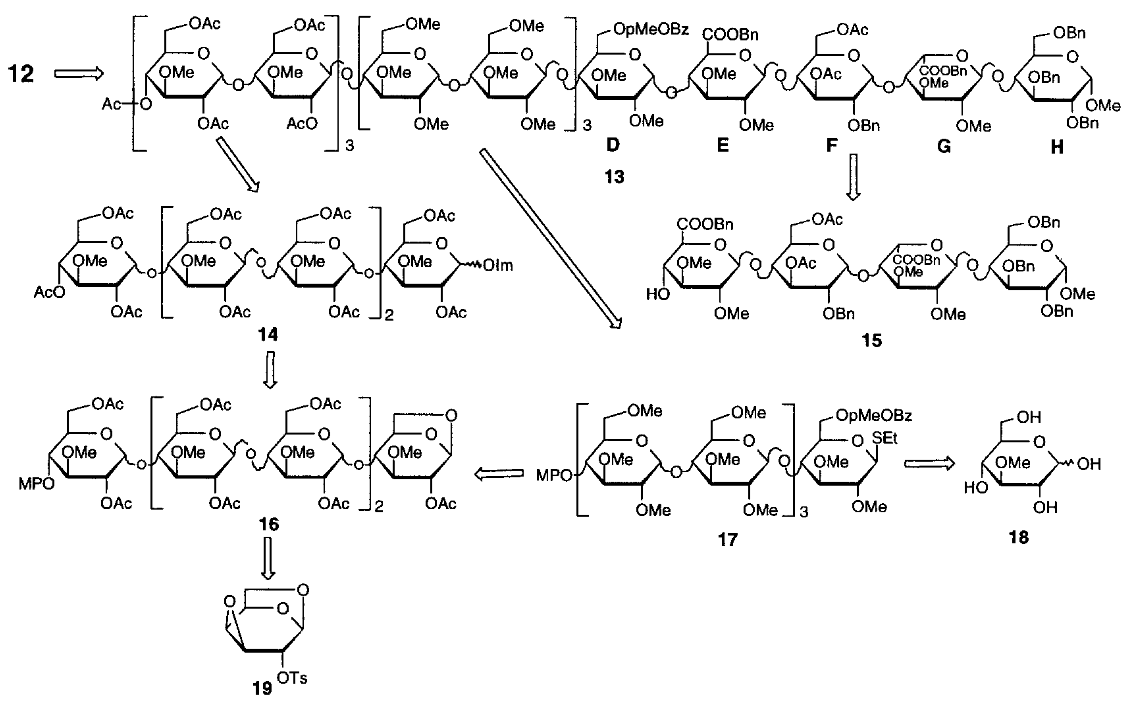

Figure 3 Retrosynthetic analysis of compound 12.

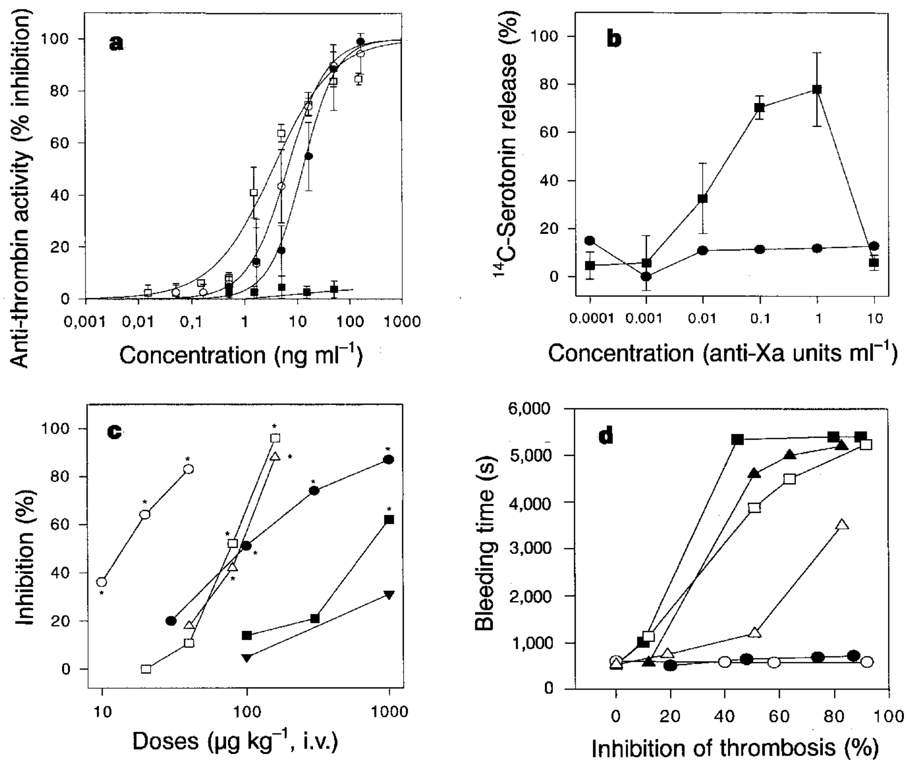

Figure 4 Biological properties of compound **12**. **a**, Inhibitory activity for thrombin. Human thrombin was incubated with antithrombin in the presence of compound **12** (circles) or standard heparin (squares) either with (filled symbols) or without (unfilled symbols) PF4. The percentage of inhibition of thrombin activity was calculated from three experiments performed in triplicate. **b**, Effect of compound **12** on platelet activation in the presence of plasma from HIT patients. Increasing concentrations of compound **12** (circles) or standard heparin (squares) were incubated with washed human platelets previously incubated with ^{14}C-serotonin in the presence of plasma from HIT patients. Platelet activation was measured by ^{14}C-serotonin release. Each point is the mean ± s.d. ($n = 6$). **c**, Venous and arterial thrombosis in the rat. The antithrombotic activity of compound **12** (circles), standard heparin (squares) or low-molecular-mass heparin (triangles) was determined in venous (unfilled symbols) and arterial (filled symbols) models of thrombosis. Each point is the mean of 10 animals per group. Grouped data were analysed for significance by comparison with the vehicle-treated group using the Mann–Whitney test with Holm–Bonferoni adjustment; $^{*}P < 0.05$. **d**, Bleeding time in the rat. Increasing doses of compound **12** (circles), standard heparin (squares) or low-molecular-mass heparin (triangles) were administered i.v. to rats. Results are expressed as mean bleeding times as a function of the antithrombotic activity of the considered compound in a venous (unfilled symbols) or arterial thrombosis model (filled symbols). Each point is the mean of 10 animals per group.

following considerations: (1) a pentasaccharide sequence (an A-domain) is required to bind and activate antithrombin towards factor Xa and thrombin inhibition; (2) the T-domain must be two to three disaccharides long[24]; (3) a chain length of 17 saccharide units is required for notable thrombin inhibition; and (4) the six or eight units of the central saccharide in compound **9** are not critically involved in the interaction either with antithrombin or with thrombin, and the charges on these units might thus be suppressed without affecting the anticoagulant activity. This analysis, which was supported by modelling studies of the ternary antithrombin/thrombin/heparin complex[10], led us to synthesize (Fig. 3) compound **12** (Fig. 2), which comprises an A-domain having a very high affinity for antithrombin[21] and a sulphated hexasaccharide as a T-domain, separated by a neutral methylated hexasaccharide.

It has been reported that a heparin fragment must be an 8-mer saccharide or a larger fragment[25] to get significant interaction with PF4. But the two charged domains in our structure are below this critical length. As might be expected from this observation, the thrombin inhibitory potency of compound **12** (Table 1, Fig. 4) was not affected by PF4, even when added at a very high concentration ($100\,\mu g\,ml^{-1}$; Fig. 4a). This may be the reason why compound **12** did not activate platelets in the presence of plasma from HIT patients (Fig. 4b), suggesting that it will not induce thrombocytopaenia. This may similarly explain why we observed an increased antithrombotic activity compared to heparin and the other synthetic derivatives described here. Thus, in models of both venous and arterial thrombosis, compound **12** was 5- to 10-fold more potent than standard heparin and low-molecular-mass heparin (Table 1, Fig. 4c). Another aspect of such a strong decrease of nonspecific interactions with basic proteins (such as von Willebrand factor and fibrinogen, the interaction that may participate in the haemorrhagic activity of heparin) is the lack of haemorrhagic activity of compound **12** compared with standard and low-molecular-mass heparin (Fig. 4d). The reduction in bleeding time observed in this experimental model may well augur a lower haemorrhagic effect observed in patients with this compound, compared with standard and low-molecular-mass heparin. Finally, from a pharmacokinetics standpoint, the compounds described here had a very simple elimination profile compared with that of standard heparin and low-molecular-mass heparin. This is probably related to the limited number of biological molecules with which they can interact, but also the structural homogeneity of these compounds obtained by chemical synthesis.

Taken together, our results demonstrate that substitutes for heparin, endowed with potent antithrombotic activity but devoid of its major side effects, can be obtained by chemical synthesis. Clinical trials will tell whether such synthetic mimetics can replace heparin, which is more than half a century old. ☐

Methods

Oligosaccharide synthesis. The general strategy for the synthesis of the sulphated oligosaccharides **1**–**12** relied on elaboration of the appropriate carbohydrate backbone adequately substituted by protective groups. After removal of the protective groups, the free hydroxyl groups were sulphonated using triethylamine-sulphurtrioxide complexes. High-field ^{1}H-nuclear magnetic resonance (500 MHz), electrospray ionization mass spectrometry, and capillary electrophoresis were used to check the structure and the homogeneity of the compounds that were 90–97% pure. Compounds **1**–**7** were efficiently obtained from a single disaccharide building-block[17]. A retrosynthetic analysis of the synthesis of compound **12** is shown in Fig. 3. The fully protected oligosaccharide **13** is a synthetic equivalent of **12**. It was obtained from tetrasaccharide **15**, which was glycosylated at position $H\text{-}4'''$ by reaction with the thioglycoside glycosyl donor **17**. After selective removal of the *p*-methoxyphenyl protecting group, a new glycosylation reaction using the imidate **14** was carried out, which delivered **13**. To minimize the number of steps, **14** and **15** derived from the same hexasaccharide precursor **16**, the key synthon of this synthesis. A *p*-methoxybenzoyl ester was chosen to protect position 6 of the D unit in **17** because we observed that such an ester strongly orientated a glycosylation reaction towards the formation of the α-anomer, when a benzyl ether was present at position 2 of glucose. The choice of a *p*-methoxyphenyl ether to protect position four of the non-reducing end unit of **16** was dictated by the many different types of reaction conditions the temporary protective group at this position had to withstand. The synthesis of **8**–**11** used a similar route but did not require the methylated synthon **17**.

Inhibitory activity for thrombin. Purified human α-thrombin ($2\,U\,ml^{-1}$) was incubated for 1 min with human antithrombin ($0.25\,U\,ml^{-1}$) at 37 °C in the presence of the various compounds with or without $10\,\mu g\,ml^{-1}$ human PF4. To measure the residual thrombin activity, S-2238 chromogenic substrate was added ($100\,\mu M$), the reaction was stopped after 1 min and the absorbance at 405 nm (A_{405}) was read. The percentage of inhibition of thrombin activity was calculated from three experiments performed in triplicate.

Inhibitory activity for factor Xa. Anti-factor Xa activities were determined by a modification of the Teien and Lie procedure as described[18]. Briefly, human factor Xa ($2.4\,nkat\,ml^{-1}$) was incubated for 2 min with human antithrombin ($0.17\,U\,ml^{-1}$) at 37 °C in the presence of various concentrations of the oligosaccharides in Tris/maleate 20 mM buffer pH 7.4, NaCl 150 mM. To measure the residual factor Xa activity, S-2222 (dissolved in 50 mM Tris-HCl buffer, pH 8.4, NaCl 175 mM, EDTA 27.5 mM, polybrene $1\,mg\,ml^{-1}$) was added (0.25 mM final). The reaction was stopped 2 min later by the addition of a 50% aqueous acetic acid solution and the A_{405} was read on a spectrophotometer. The activity per mg of the compounds was determined by comparison with a calibrated standard.

Affinity for human antithrombin. Affinity of the oligosaccharides for human antithrombin was determined by fluorescence as described[26] using a Perkin Elmer LS-50 type spectrofluorometer at a λ excitation of 280 nm and a λ emission of 338 nm at 37 °C, under continuous stirring. Oligosaccharides were added into 2 ml of Tris-HCl buffer (0.01 mM, pH 7.0) containing 0.15 M NaCl and 5–60 nM antithrombin. The ratio and the concentrations of antithrombin–oligosaccharide complex were calculated and dissociation constants (K_d) were determined from Scatchard plots using RS/1 software.

Platelet activation in the presence of plasma from HIT patients. Increasing concentrations of the various compounds were incubated with washed human platelets (3×10^5 platelets per μl) previously incubated with ^{14}C-serotonin ($0.05\,\mu Ci\,ml^{-1}$) in the presence of plasma from HIT patients. After 60 min of incubation, platelet activation was measured by ^{14}C-serotonin release as described[27].

Venous and arterial thrombosis in the rat. To determine their venous antithrombotic activity, the compounds were administered intravenously (i.v.) to rats 5 min before venous thrombosis induced in the vena cava by a combination of stasis and tissue factor ($1\,ng\,kg^{-1}$, i.v.) as described previously[18]. Their arterial antithrombotic activity was determined in an arterio-venous shunt model as described[18,19]. Grouped data were analysed for significance by comparison with the vehicle-treated group using the Mann–Whitney test with Holm–Bonferoni adjustment taking $P < 0.05$ to indicate a significant difference.

Bleeding time in the rat. Increasing doses of the various compounds were administered i.v. to rats 15 s before the transection of the tip of the tail of pentobarbital-anaesthetized rats. Bleeding time was then measured as described[18].

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Correspondence and requests for materials should be addressed to J.-M.H. (e-mail: jean-marc.herbert@sanofi.com).

β-Catenin regulates expression of cyclin D1 in colon carcinoma cells

Osamu Tetsu & Frank McCormick

University of California, San Francisco, School of Medicine,
Cancer Research Institute, San Francisco, California 94143-0128, USA

Mutations in the adenomatous polyposis coli (APC) tumour-suppressor gene occur in most human colon cancers[1]. Loss of functional APC protein results in the accumulation of β-catenin[2]. Mutant forms of β-catenin have been discovered in colon cancers that retain wild-type APC genes[3,4], and also in melanomas[5], medulloblastomas[6], prostate cancer[7] and gastric[8] and hepatocellular[9,10] carcinomas. The accumulation of β-catenin activates genes that are responsive to transcription factors of the TCF/LEF family, with which β-catenin interacts[11–15]. Here we show that β-catenin activates transcription from the *cyclin D1* promoter, and that sequences within the promoter that are related to consensus TCF/LEF-binding sites are necessary for activation. The oncoprotein p21*ras* further activates transcription of the *cyclin D1* gene, through sites within the promoter that bind the transcriptional regulators Ets or CREB. Cells expressing mutant β-catenin produce high levels of cyclin D1 messenger RNA and protein constitutively. Furthermore, expression of a dominant-negative form of TCF in colon-cancer cells strongly inhibits expression of cyclin D1 without affecting expression of cyclin D2, cyclin E, or cyclin-dependent kinases 2, 4 or 6. This dominant-negative TCF causes cells to arrest in the G1 phase of the cell cycle; this phenotype can be rescued by expression of cyclin D1 under the cytomegalovirus promoter. Abnormal levels of β-catenin may therefore contribute to neoplastic transformation by causing accumulation of cyclin D1.

Genes that might be targets for transcriptional activation by the β-catenin/TCF complex have been identified in various organisms. These include the mouse E-cadherin gene[16], the connexin 43 gene[17], *Drosophila ultrabithorax*[18], *Xenopus siamois*[19] and *nodal-related 3* (ref. 20). More recently, the c-*myc* gene has been implicated as a target of the APC pathway in colon cancer[21]. c-*myc* expression is inhibited by expression of APC, and the c-*myc* promoter contains elements that bind TCF *in vitro*. Also, many colon carcinomas show high levels of c-*myc* expression. However, it is not clear whether β-catenin activates c-*myc* directly, nor to what extent c-*myc* expression contributes to malignant growth. To identify candidate target genes of β-catenin-mediated transcription, we searched the GenBank database for human genes involved in controlling cell growth whose promoter region contains the core TCF/LEF-binding site 5′-A/T A/T CAAAG-3′ (ref. 14). Through a series of computer searches, we identified four candidate target genes, *cyclin D1*, *cyclin A*, *cdc2* and *cdc25C*. Of these, *cyclin D1* is of particular interest because cyclin D1 is over-expressed in many colon carcinomas, although the gene is rarely amplified[22] and inhibition of *cyclin D1* expression causes growth arrest in colon carcinoma cell lines[23]. The core TCF/LEF consensus site does not occur in promoter regions of other cyclins, including *cyclin D2* and *cyclin D3*, *cyclin E*, *cyclin G*, nor in other cyclin-dependent kinases whose promoter regions have been sequenced, such as *cdk2*, *cdk4*, *cdk5*, *cdk6* and *cdk7*.

To determine whether β-catenin regulates the transcription of these genes, we transfected luciferase reporter genes into HeLa cells and measured their response to β-catenin. Two forms of β-catenin expression vector were tested. One encodes wild-type β-catenin, the other encodes a mutant form that lacks amino acids 29–48 and

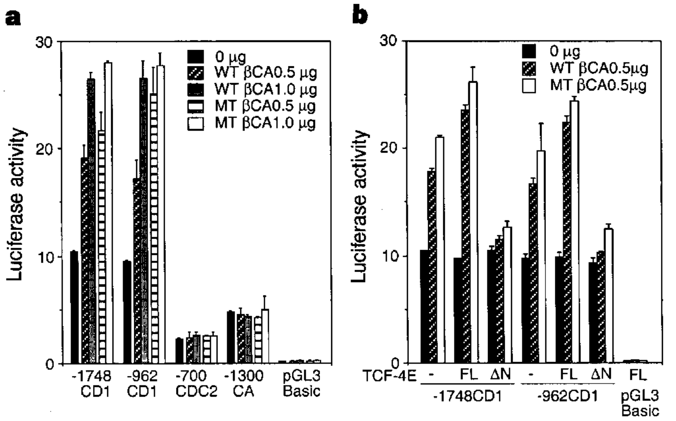

Figure 1 β-Catenin activates the *cyclin D1* promoter. **a**, HeLa cells were transfected with promoters from *cyclin D1*, *cdc2* or *cyclin A* driving luciferase transcription, and 0.5 or 1 μg of wild-type (WT) or mutant-type (MT) β-catenin (βCA) expression plasmids. Two forms of *cyclin D1* promoter were used, one consisting of 1,748 bp from the transcriptional start (−1748CD1), the other of 962 bp (−962CD1). Empty pcDNA3 (Invitrogen) was added to 2 μg of plasmid DNA. 50 ng of pRL-TK *renilla* luciferase reporter construct (Promega) was co-transfected to each sample to normalize transfection efficiency. The ratio of reporter luciferase activity to control *renilla* luciferase activity is indicated. The assay was performed 24 h after transfection. All experiments are expressed as mean ± s.d. of triplicate cultures. **b**, Effects of dominant-negative TCF-4E on β-catenin-dependent *cyclin D1* promoter activity. Full-length (FL) TCF-4E complementary DNA was subcloned into Myc-tagged pcDNA3 from TCF-4E pcDNAI. N-terminal-deleted (ΔN) Myc-tagged TCF-4E pcDNA3 was generated by PCR-based site-directed deletion mutagenesis.